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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

22469

PATENT TRADEMARK OFFICE

Art Unit :
Examiner :
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Inventor : Michèle Amouyal
Title : CONSTRUCT OF RECOMBINANT NUCLEIC ACIDS CIRCULARIZED BY DNA COMPACTING AGENTS

Docket No.: 1231-01
Confirmation No.: 2241

Dated: October 4, 2001

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, DC 20231

Sir:

Prior to action on the merits, we respectfully request consideration of the following amendments and remarks:

Version with Markings to Show Changes to the Title

~~CONSTRUCTION OF~~ CIRCULARIZED RECOMBINANT NUCLEIC ACIDS AND PROCESS FOR CONSTRUCTING THEM BY MEANS OF DNA COMPACTING AGENTS

Version with Markings to Show Changes to the Specification

Kindly enter the following changes to the Specification:

Prior to the first paragraph on page 1, please insert the following:

RELATED APPLICATION

This is a continuation of International Application No. PCT/FR00/0004, with an international filing date of January 4, 2000, which is based on French Patent Application No. 99/00148, filed August 1, 1999.

FIELD OF THE INVENTION

Please replace paragraph [0003] with the following paragraph:

[0003] The construction of DNA vectors intended to be transferred into prokaryote or eukaryote cells according to this invention envisages all *in-vitro* and *in-vivo* uses of these sequences such as analysis of the biological effects of the DNA sequence (effect of the DNA or of its expression), expression of RNA, expression of proteins, amplification of hybridization probes for medical diagnosis, cell or gene therapy, vaccination, etc.

Please replace paragraph [0004] with the following heading and paragraph:

BACKGROUND

[0004] Recombinant vector construction, comprising insertion of a DNA fragment in a vector, includes an *in-vitro* recombinant vector reclosure step. In order for reclosure to take place, the ends of the finished vector must be in close proximity to each other. These ends move within a sphere with a radius equal to the length of the fragment and with one of the ends as its center. The greater the size of the finished vector, the greater the volume of this sphere. Consequently, the probability of conjunction of the two ends decreases with the length of the finished vector.

Please replace paragraph [0005] with the following paragraph:

[0005] ~~With regard to insertion~~Insertion of the fragment in the vector~~, it takes place~~ occurs if the probability of conjunction between the ends of the insert and those of the original vector is high. Thus, insertion is dependent on the concentration of ends.

Please replace paragraph [0007] with the following paragraph:

[0007] The very first generation vectors are vectors of bacterial origin derived from plasmids. Other vectors take into account more precisely the specificities of the eukaryote genes, in particular their size and have been developed to allow cloning of large pieces of DNA. This is the case ~~of the~~for cosmids, hybrid compounds of phage l and plasmids reproducing in *E. coli*, and ~~the~~ YACs whose host organism is yeast.

Please replace paragraph [0008] with the following paragraph:

[0008] The cosmids enable insertion up to 45 kb, the YACs up to 1000 kb. These vectors are intended for analysis of genomic DNA libraries or chromosomal analysis. Cosmids and YACs are responsive to the requirements of these analytical techniques and allow a search for a broad insertion range from DNA libraries. Their efficacy is linked to the range of different fragments that they can receive. Their use is burdensome if ~~it is a question of~~ only ~~inserting~~insertion of a specific fragment and ~~amplifying it~~amplification of it are desired. Thus, in order to preserve their stability, the insertion vector must ~~conserve~~have a minimum size ~~(~~of 33 kb for the cosmids~~,~~ and 150 kb for the YACs~~)~~. However, handling such lengths of DNA is fraught with numerous problems (breaks, cuts, etc.), ~~whereas~~and the sequence under consideration is frequently of smaller size, only reaching several kb when complementary DNA is preferred to genomic DNA. Moreover, replication in *E. coli* of vectors of large size such as cosmids as well as the stability of

YACs in yeast are limited, which leads to sequence modifications. In the case of the cosmids, the efficacy of the DNA packaging and of the infection by the phage l compensates for this disadvantage to a certain extent.

Please replace paragraph [0009] with the following paragraph:

[0009] Another insertion technique comprises the use of adjuvants of a chemical nature such as polyethylene glycol (Zimmerman S. B. and Pheiffer B. H., Proc. Natl. Acad. Sci. USA, 80, 5852 (1983)) or hexamine cobalt chloride (Maniatis et al., Molecular Cloning / A Laboratory Manual, second edition, 1989) for straight-end ligations. These ligations are not as easy to implement as cohesive-end ligations, and require higher concentrations of DNA and ligase. The purpose of these chemical adjuvants is to promote aggregation between insert and vector (intermolecular reaction) and to decrease the concentrations of DNA and ligase; ~~they do not apply~~ these methods do not apply to the reclosure step (intramolecular ligation reaction) and to the construction of vectors of large size. The term "vector of large size" is understood to mean a vector of at least 10 kb into which is integrated an insert of multiple kilobases.

Please replace paragraph [0010] with the following paragraph:

[0010] ~~At~~The classical technique for construction of first generation vectors (E. coli derived plasmids) is effective for moderate sizes (several thousands of base pairs) but gradually loses its efficacy for larger vectors (more than 10kb). Moreover, at ~~At~~ present, there is no technique which facilitates the circularization and preparation of vectors which is effective for vectors of moderate sizes~~,~~ -- several thousand of base pairs~~,~~ -- as well as for larger sizes~~,~~ -- ~~above~~more than 10 kb. Third, no known ligation adjuvants presently employed are proteins naturally designed to condense the DNA or have the effect of

promoting the insertion of fragments of large size or reclosure of the recombinant.

Please delete paragraphs [0011] and [0012].

Prior to paragraph [0013], please insert:

BRIEF DESCRIPTION OF THE INVENTION

Please replace paragraph [0015] with the following paragraph:

[0015] In fact, one ~~of the values~~benefit of the invention is in the domain of cloning which generally includes a prior step of *in-vitro* cyclization of the recombinant. The ~~efficacy of the cyclization depends on the size of the recombinant and is improved by compaction of the DNA. In fact, vector construction includes an *in-vitro* vector closure step. The~~closure of the recombinant fragment (linearized initial vector and insertion) can be meant as a DNA cyclization. Like DNA cyclization, the efficacy of ~~this~~vector closure depends on the size of the recombinant DNA fragment and is improved by DNA compaction.

Please insert the following paragraph [0016.1](formerly paragraph [0019]):

[0016.1] The phrase "by any suitable means" in step (b) should be understood to mean the transfer of the ligation products into a cellular medium suitable for DNA cloning, e.g., *E. coli* or yeast, in the presence of an antibiotic such as ampicillin or tetracycline, if the vector carries an antibiotic resistance gene, and a test for the presence of the insert, for example, in the case of the control gene lacZ, hydrolysis of the x-gal compound by the b-galactosidase produced.

Please replace paragraph [0017] with the following paragraph:

[0017] Thus, one application of the process of the invention is the cloning of an insert in a vector under the conditions defined above, ~~enabling~~ advantageously enabling production of a recombinant vector of large size.

Please insert the following paragraphs (formerly paragraphs [0053]-[0057]):

[0018.1]Fig. 1 shows the complexation of the protein to the fragment fR4 under ligation conditions followed by retardation of the electrophoretic migration of the fragment in 0.4% agarose gel.

- 1a: Trial 1
- 1b: Trial 4
- 1c: Trial 1 with purified protein H1 (example 2)

[0018.2]Fig. 2 shows the digestion by EcoRI of the three recombinants R4-LZ with insert obtained in trial 11.

[0018.3] Fig. 3 shows the digestion by EcoRI of 6 of the 20 recombinants K-LZ (example 1b).

[0018.4] Fig. 4 shows the augmentation of the quantity of ligation products in the presence of histones, 0.4% agarose gel.

[0018.5] Fig. 5 shows the sensitivity test to the nucleases possibly present in the histone preparation.

Please delete paragraph [0019].

Please delete paragraph [0021].

Please replace paragraph [0022] with the following heading and paragraph:

DETAILED DESCRIPTION

[0022] According to the invention, ligation of the insert and of the original vector is implemented in the presence of DNA compacting products. The insertion vector contains a replication origin and, advantageously, a selection gene for growth and selection in the cell type under consideration. According to one preferred implementation, the vector is a nonviral vector, albeit capable of containing viral elements.

Please replace paragraph [0024] with the following paragraph:

[0024] The DNA compacting products are proteins or any agents exhibiting the same properties, and more particularly the histone proteins or related proteins. Histones are the most abundant proteins in the nucleus; they are of small size (11 to 25 kDa) and of a very basic nature (pH > 10). There are five types of histones which are referred to as H1, H2A, H2B, H3 and H4, respectively. These five types are found with variants in all of the eukaryotes (with the exception of H1 which does not appear to exist in yeast, and which is replaced by histone H5 in certain organisms). H2A, H2B, H3 and H4 are found *in vivo* in octamer form. The DNA coils twice around the octameric core so as to form a nucleosomic structure. The histone H1 does not participate in this nucleosome core but serves to seal the DNA around the octamer. In the eukaryote cell, the compaction of the DNA by the histones has the effect, notably, of bringing close to each other two transcriptional regulation sites which are situated remotely from each other on the chromosome, and to enable formation of a chromatin loop by direct interaction between these sites. Thus, the expression of a gene can be controlled remotely by these genes (Amouyal, Biochimie (1991), 73, p. 1261-1268).

[0024.1] In addition to the histones, the DNA compaction agent can be selected from all

the proteins known to compact DNA, especially the viral or phage envelope proteins, the bacterial chromoid proteins (HU, H-NS, etc.), the non-histone chromosomal proteins, the HMGs, etc., all mixtures of these compounds, or any derivative thereof.

Please delete paragraph [0026].

Please replace paragraph [0027] with the following paragraph:

[0027] The DNA compaction agent is preferably either a mixture of histones or a mixture of the aforementioned compaction agents.

Please replace paragraph [0028] with the following paragraph:

[0028] The inventor has demonstrated that a mixture of histones or an isolated histone lead to similar results. The histone H1, of different structure and not being a histone forming the octamer but rather a sealing histone, used by itself, however, does not appear to yield results as good as the other histones. This is explained by the fact that, because of its different structure, histone H1 does not bind to linear DNA but rather preferably to supercoiled DNA (Van Holde et al., Biophysical Journal, 1997, 72, p. 1388-1395).

Please delete paragraph [0029].

Please replace paragraph [0035] with the following paragraph:

[0035] This compaction agent concentration (C) can be expressed in ~~mg~~milligrams (mg) of proteins per nanogram (ng) of total DNA contained in the ligation mixture ~~and by~~per base pair (bp) of recombinant. The concentration is dependent on the length of DNA to be ligated and the inventor has defined a multiplicity of laws, each corresponding to the

protein agents employed~~:~~.

Thus for the natural mixture of histones Sigma,

(C) = $1.5 \cdot 10^{-11}$ mg/ng DNA/bp recombinant

for the histone H2B Sigma,

(C) = $1.5 \cdot 10^{-12}$ mg/ng DNA/bp recombinant.

Please replace paragraph [0036] with the following paragraph:

[0036] By extrapolation, the inventor defined the following law (I) which can be applied to all of the compacting proteins employed:

(C) = 10^{-x} mg/ng DNA/bp recombinant

in which x is between 8 and 15 inclusively.

The value x is a function of the nature of the compaction agent employed.

Please replace paragraph [0037] with the following paragraph:

[0037] In practice, the efficacy of the cloning is always improved if the concentration is within a range ~~encompassing~~encompassed by the value ~~thereby~~ defined above and extending from 20 to 1000% of that value, and preferably between 33 and 200% of this value.

Please replace paragraph [0038] with the following paragraph:

[0038] The process according to the present invention is thus characterized in that the concentration(C) of compaction agent is determined by the following law (II):

(C) = (10^{-x} mg/ng DNA/bp recombinant) x Y

in which:

x is comprised between 8 and 15, preferably between 10 and 12, and

Y varies between 0.2 and 10, preferably between 0.33 and 2.

Please replace paragraph [0043] with the following paragraph:

[0043] Prior to ligation, the ~~protein~~compaction agent is put into solution in the ligation buffer —possibly containing glycerol—(or diluted in the ligation medium, if the agent is provided in solution form). It is incorporated in the ligation medium in the proportions defined by the law specified above.

Please replace paragraph [0044] with the following paragraph:

[0044] In a preferred manner, the kit includes a stabilizing agent incorporated in the ligation medium at the same time as the compaction protein, which stabilizing agent is designed to prevent the denaturation, aggregation and/or adsorption of the compaction protein on the walls of the reaction tube at these strong dilutions. Thus, a kit according to the invention comprises:

- a ligation buffer,
- a ligase,
- a compaction agent,
- possibly a stabilizing agent for the protein.

Please replace paragraph [0045] with the following paragraph:

[0045] In a preferred manner, ~~this dilution buffer~~the stabilizing agent is glycerol or any compound presenting the same characteristics.

Please replace paragraph [0049] with the following paragraph:

[0049] The applications of the invention are, of course, to be found in the field of cloning, but also in the domain of gene therapy, most especially in the context of genetic vaccination. Thus, the invention pertains more specifically to a circularized recombinant nucleic acid of large size greater than several kilobases constituted by a vector and an insert. In a first form of implementation of the invention intended for gene therapy, said insert comprises one or more cDNA <u>sequences</u> coding for one or more proteins required for the correction of a genetic deficiency, placed under the control of sequences enabling their *in-vivo* expression.

Please replace paragraph [0050] with the following paragraph:

[0050] In a second form of implementation of the invention intended for genetic vaccination, said insert comprises one or more DNA <u>sequences</u> coding for one or more antigens, placed under the control of sequences enabling their *in-vivo* expression. More specifically, said insert comprises one or more, and preferably the totality, of the DNA sequences coding for antigens capable of inducing an immune reaction.

Please delete paragraph [0051].

Please delete paragraphs [0053]-[0057].

Please amend the heading after paragraph [0058] as follows:

<u>Example 1a: Cloning a recombinant of 12,034 bp: Recombinant R4-LZ</u>

Please replace paragraph [0062] with the following paragraph:

[0062] The 8,159-bp fragment (fR4) contains the replication origin colE1 and the ampicillin-resistance gene of *E. coli*. It is separated from the other fragments by electrophoretic migration on low-melting-point agarose gel (Seaplaque LMP agarose, FMC Bioproducts), excision and extraction of the corresponding band.

– Fragment of 3,875 base pairs: fLZ

Please replace paragraph [0064] with the following paragraph:

[0064] By digestion at the unique sites SpeI (position 29) and SalI (position 3904) followed by electrophoretic separation, the 3,875-bp fragment (fLZ)containing the gene lacZ under control of the bacterial promoter was separated from the 2,331-bp fragment.

Please replace paragraph [0068] with the following paragraph:

[0068] When necessary, the compaction protein is added to the mixture of fragments at the desired concentration. The T4 DNA ligase is added after incubation in a period of time between 0 to 20 minutes, more particularly between 3 and 5 minutes.

Please replace paragraph [0069] with the following paragraph:

[0069] The E.coli strain DH5a is a strain deficient in protein RecAl, which does not promote recombinations and rearrangements of the DNA within the cell (notably, the plasmids remain in monomer form while deletions are avoided, pages 4-13 of Maniatis).

Please replace paragraph [0071] with the following paragraph:

[0071] A first histone concentration value was determined by calculating the quantity of the natural mixture of histones assumed to be necessary for the formation of one

nucleosome every 200 base pairs, and choosing deliberately to only take a part of the amount of protein thereby determined (one fourth in this example) so as to avoid saturation of the fragment in protein. ~~The law~~Law III derived from this is the following:

(C) = $1.2 \cdot 10^{-11}$ mg of histone mixture/ng of DNA/bp of recombinant ...(III).

Please replace paragraph [0073] with the following paragraph:

[0073] The law, which was readjusted on the basis of these gels and which was subsequently employed, is very close to the calculated value:

(C) = $1.5 \cdot 10^{-11}$ mg of the histone mixture / ng of DNA / bp of recombinant ...(IV).

Please replace paragraph [0074] with the following paragraph:

[0074] Each ligation was performed in the presence of three concentrations of compaction proteins, the quantity derived from ~~this law two~~Law IV and quantities encompassing this quantity and corresponding to 50 and 200% of the calculated quantity, respectively.

Please replace paragraph [0075] with the following paragraph:

[0075] On these gels, we have:

- Track 1: Fragment fR4 without compaction proteins (except in the gel corresponding to trial 4),
- Track 2: Fragment fR4 + 0.025 μg of compaction protein,
- Track 3: Fragment fR4 + 0.05 μg of compaction protein,
- Track 4: Fragment fR4 + 0.1 μg of compaction protein,
- Track 5: Fragment fR4 + 0.2 μg of compaction protein (trial 4).

Please replace paragraph [0081] with the following paragraph:

[0081] The ~~transformations~~transformation of *E. coli* ~~were~~was implemented with the ligation mixture containing only one of these two fragments, in the presence of or absence of histones, to confirm the electrophoretic purity of the fragments and the absence of reclosure within the cell. ~~A gel retardation assay was performed on the fragment fR4 prior to each ligation with this fragment in order to monitor the complexation of the DNA by the protein. Separation was performed on standard 0.4% agarose gel. This gel assay was performed under the same conditions as the ligation experiments in 20 μl deposited on gel and is shown in figure 4, in which:~~The composition of the ligation mixture was analyzed prior to cellular transformation by standard gel electrophoresis. This analysis indicates that the addition of histones favors the obtention of ligation products prior to cellular transformation. For this purpose, the 20 μl of ligation mixture were deposited on a standard 0.4% agarose gel. The results are shown on Figure 4, in which:

- Track 1: ligation mixture (recombinant R4-LZ) without histones (fragments fR4-8159 bp and fLZ-3875 bp) ~~arrows~~
- Track 2: ligation mixture in the presence of 0.025 μg of histones
- Track 3: ligation mixture in the presence of 0.05 μg of histones
- Track 4: purified recombinant R4-LZ
- Track 5: 1-kb marker (Gibco BRL)
- Track 6: marker f_x174/HaeIII (Gibco BRL)

Please replace paragraph [0082] with the following paragraph:

[0082] The first 13 trials were performed on the basis of the quantity of compaction protein determined by gel retardation assay from the complexation of the fragment fR4 ~~by itself rather than the law stemming from these experiments~~while they should have been

performed on the basis of the complexation of the total recombinant (fR4 + fLZ) fragment. However, the size ~~(8159 bp)~~ of the fragment fR4 represents only 2/3 of the total size of the recombinant. By employing ~~this~~these values for the ligations, only 2/3 of the value calculated according to the law was ~~only followed by two thirds~~ used (2/3 law). The more recent trials, which were also the more successful, were performed by taking into account the quantity of compaction protein (1/3 more, thus the exact value) that needed to be added to complex the entire vector and insert unit in the same manner as the fragment by itself.

Please replace paragraph [0085] with the following paragraph:

[0085] II.1. ~~Trials 1-6, 9-13: 2/3 law~~2/3 Law (Trials 1-6, 9-13) versus Exact Law (Trials 14 and 15)

- Trials 4 and 4': 4 quantities of compaction protein were tested: 50, 100, 200, 400%
- Trial 7: quantities of compaction protein 3 times higher than Trial 4 (150, 300, 600%)
- Trial 8: quantities of compaction protein 10 times higher than Trial 4 (500, 1000, 2000%)

(to compensate for a possible complexation deficit due to a lower concentration of DNA)

- Trials 14 and 15: exact law

Please replace paragraph [0086] with the following paragraph:

[0086] II.2. Effect of storage and freezing of the compaction protein:

- Trial 2: dilution of the compaction protein from a stock-solution of 100 mg/ml in

the dilution buffer containing 50% glycerol, stored at -20°C to test the effect of storage. For all of the other trials, the protein solution was freshly prepared from the lyophilized powder.

Please replace paragraph [0090] with the following paragraph:

[0090] II.6. Presence of glycerol in the dilution buffer

- Trials 9, 10, 11', 12', 12''', 14, 15

Glycerol is usually added to the storage buffer, as above, to prevent denaturation and aggregation of ~~the~~ proteins during ~~the~~ successive freezing-thawing cycles. Thus, 0.1% of glycerol was added to the compaction protein dilution buffer (ligation buffer) so as to prevent aggregation or denaturation of the compaction protein when it was put into solution, as well as to avoid the loss of compaction protein on the tube walls in the final dilutions. In trial 11', glycerol was added on a delayed basis (after the first ligation series of trial 11, i.e., after approximately ten minutes).

Please insert paragraph [0090.1] (formerly paragraph [0096]):

[0090.1] The presence of glycerol when the protein is put into solution appears to be beneficial to the cloning efficacy in the case of the natural histone mixture. It does not appear to be especially beneficial with the isolated histones (H1 or H2B, example 2). That is why the kit can contain (irrespective of the protein employed) a dilution buffer containing glycerol (or any other agent with the same properties) as well as a dilution buffer without glycerol (which is also the ligation buffer).

Please replace paragraph [0092] with the following paragraph:

[0092] ~~We always selected a part of the~~ In each trial, recombinants not possessing the anticipated sequence by cloning in *E. coli* were selected~~, even in the absence of protein~~

~~adjuvants~~. These defects are generated at the time of *in vitro* ligation or by incorrect replication of the DNA by the cell.

Please replace paragraph [0094] with the following paragraph:

[0094] In order to determine whether the presence of these incorrect recombinants is due specifically to the use of ~~a~~the compaction protein preparation, especially to the possible presence of nucleases, and to the partial degradation of the DNA which could result from it, a DNA sensitivity test to the nucleases in the histone preparation employed was performed. For this test, the fragment fR4 or the plasmid pR4 was incubated with the quantities of protein indicated on tracks 3 to 10. The results are illustrated in figure 5, in which:

- Track 3: fragment fR4 (500 ng)
- Track 4: fragment fR4 + 0.025 μg of histones
- Track 5: fragment fR4 + 0.05 μg of histones
- Track 6: fragment fR4 + 0.1 μg of histones
- Track 7: plasmid pR4 (500 ng)
- Track 8: plasmid pR4 + 5 μg of histones
- Track 9: plasmid pR4 + 0.025 μg of histones
- Track 10: plasmid pR4 + 0.05 μg of histones
- Tracks 11, 12, 13: like tracks 8, 9, 10. Incubation at 20°C for 1.5 hours rather than 20 hours.
- Tracks 1 and 14: 1-Kb marker (Gibco BRL)
- Track 2 and 15: marker f_x174/HaeIII (Gibco BRL)

No nuclease digestion was detected under these conditions.

Please delete paragraph [0096].

Please replace paragraph [0098] with the following paragraph:

[0098] Example 1b: Cloning of a 6,785-bp ~~fragment~~recombinant: Recombinant K-LZ

I – Material and Methods

I.1 Fragments

- Fragment of 2910 base pairs: fK.

Please replace paragraph [0102] with the following paragraph:

[0102] The quantities of compaction protein employed corresponded to 33, 67 and 134% of the quantity indicated by the law specified above (2/3 law).

Please replace paragraph [0104] with the following paragraph:

[0104]The usual technique is still valid for this 6,785 bp construct, in the absence of histones, while it was not anymore for the larger recombinant R4-LZ, under the same condition. However, the addition of histones also increases the cloning efficacy~~The presence of histones at the time of ligation also improves the cloning efficacy~~.

Please replace paragraph [0111] with the following paragraph:

[0111] Example 2b: ligation in the presence of histone H2B

In this case, the protein (M = 13,774) represents one tenth of the mixture (M = 130,268), and the law becomes:

(C) = $1.5 \cdot 10^{-12}$ mg H2B/ng DNA/bp of recombinant.

Version with Markings to Show Changes to the Claims

Please cancel Claims 1-10 without prejudice and without disclaimer of the subject matter contained therein.

Kindly add the following new claims:

11. (New) A process for preparing circularized recombinant nucleic acids from a vector and an insert comprising the steps of:

producing circularized recombinant nucleic acid by ligating a DNA insert and a DNA vector in the presence of a DNA compaction agent;

and

selecting said circularized recombinant nucleic acid.

12. (New) The process according to Claim 11, wherein the size of said circularized recombinant nucleic acid is greater than 10 kb.

13. (New) The process according to Claim 11, wherein said selection comprises the steps of:

transferring said circularized recombinant nucleic acid into a cellular medium,

cloning said circularized recombinant nucleic acid, and

testing for presence of said insert in said circularized recombinant nucleic acid.

14. (New) The process according to Claim 11, wherein said DNA compaction agent is one selected from the group consisting of a protein, a mixture of proteins, and protein derivatives.

15. (New) The process according to Claim 11, wherein said DNA compaction agent is one selected from the group consisting of histone proteins, histone protein derivatives, viral envelope proteins, phage envelope proteins, bacterial chromoid proteins, non-histone chromosomal proteins, HMGs, derivatives of said proteins, and mixtures of said proteins and protein derivatives.

16. (New) The process according to Claim 11, wherein said ligation comprises the step of adding a ligase to a ligation medium comprising DNA in solution in ligation buffer.

17. (New) The process according to Claim 16, wherein said DNA compaction agent is added to said ligation medium prior to the addition of said ligase.

18. (New) The process according to Claim 16, wherein said DNA compaction agent is added to said ligation medium simultaneously with the addition of said ligase.

19. (New) The process according to Claim 11, wherein said DNA compaction agent is present at a concentration (C) allowing flexibility of said DNA.

20. (New) The process according to Claim 19, wherein said DNA compaction agent concentration (C) is defined by the following equation:

$$(C) = 10^{-x} \text{ mg DNA compaction agent/ng DNA/bp recombinant},$$

wherein X = 8-15.

21. (New) The process according to Claim 19, wherein said DNA compaction agent concentration (C) is defined by the following equation:

$$(C) = (10^{-x} \text{ mg DNA compaction agent/ng DNA/bp recombinant}) \times Y$$

wherein X = 8-15 and

$$Y = 0.2 - 10.$$

22. (New) The process according to Claim 16, wherein said ligation medium further comprises a stabilizing agent, wherein said stabilizing agent is capable of preventing denaturation, aggregation, and absorption of said DNA compaction agent.

23. (New) The process according to Claim 15, wherein said histone proteins are selected from the group consisting of histone H1, H2A, H2B, H3, and H4.

24. (New) A kit for preparing circularized recombinant nucleic acids from a vector and an insert comprising a ligation buffer, a ligase, and a DNA compaction agent.

25. (New) The kit according to Claim 24, wherein said DNA compaction agent is one selected from the group consisting of histone proteins, viral envelope proteins, phage envelope proteins, bacterial chromoid proteins, non-histone chromosomal proteins, HMGs, derivatives of said proteins, and mixtures of said proteins and protein derivatives.

26. (New) The kit according to Claim 25, wherein said histone proteins are selected from the group consisting of histone H1, H2A, H2B, H3, and H4.

In the Abstract

Kindly enter the following Abstract enclosed on a separate sheet.

A process for preparing circularized recombinant nucleic acids of moderate to large size by ligating an insert and a vector in the presence of a DNA compaction agent followed by selecting for the circularized recombinant nucleic acid and a kit for preparing the circularized nucleic acids in the presence of a DNA compaction agent.

Remarks

The English translation of PCT/FR00/00004 is submitted herewith. We have amended the Specification to correct minor typographical and idiomatic errors. The majority of the inserted text has simply been relocated from other parts of the specification to improve readability. Additionally, in paragraph [0045], the phrase "the dilution buffer" has been changed to "the stabilizing agent" as supported by paragraph [0046]. No new matter has been added.

We have canceled Claims 1-10 without prejudice and without disclaimer of the subject matter contained therein. New Claims 11-26 have been added.

Claim 11 is drawn to a process for preparing circularized recombinant nucleic acids by ligating an insert and a vector in the presence of a DNA compaction agent to produce a circularized recombinant nucleic acid and selecting therefor, as supported by the Specification at paragraph [0016].

Claims 12 specifies the size of the circularized recombinant nucleic acid of Claim 11 as supported in the Specification at paragraph [0018].

Claim 13 defines the selection process of Claim 11 as supported by the Specification at paragraph [0016.1].

Claims 14-15 and 23 identify the DNA compaction agent of the process as supported in the Specification at paragraphs [0023]-[0027].

Claim 16 defines the ligation process of Claim 11 as supported at paragraphs [0030]-[0031] of the Specification.

Claims 17 and 18 specify the timing of the addition of the DNA compaction agent in the process, as supported by the Specification at paragraphs [0043] and [0088], respectively.

Claim 19 specifies the requirement that the DNA compaction agent concentration in the process allow DNA flexibility as supported in the Specification at paragraph [0033].

Claims 20 and 21 define the equation for determining the DNA compaction agent concentration, as supported in the Specification at paragraph [0036]-[0038].

Claim 22 is drawn to the process of Claim 16, further having a stabilizing agent, as supported in the Specification at paragraph [0044].

Claim 24 encompasses a kit for preparing circularized recombinant nucleic acids having a ligation buffer, a ligase and a DNA compaction agent, as supported in the Specification at paragraph [0039].

Claim 25 defines the DNA compaction agent of Claim 24, as supported in the Specification at paragraphs [0023]-[0027]. Claim 26 further defines the DNA compaction agent of the kit, as supported at paragraph [0024].

As each of the new Claims is supported by the Specification, no new matter has been added.

We look forward to a prompt action on the merits and urge allowance of the Claims as amended.

Respectfully submitted,

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In the Title (Clean copy as amended)

CIRCULARIZED RECOMBINANT NUCLEIC ACIDS AND PROCESS FOR CONSTRUCTING THEM BY MEANS OF DNA COMPACTING AGENTS

<u>In the Specification</u> (Clean Copy as Amended)

Kindly enter the following changes to the Specification:

Prior to the first paragraph on page 1, please insert the following:

RELATED APPLICATION

This is a continuation of International Application No. PCT/FR00/0004, with an international filing date of January 4, 2000, which is based on French Patent Application No. 99/00148, filed August 1, 1999.

FIELD OF THE INVENTION

Please replace paragraph [0003] with the following paragraph:

[0003] The construction of DNA vectors intended to be transferred into prokaryote or eukaryote cells according to this invention envisages all *in-vitro* and *in-vivo* uses of these sequences such as analysis of the biological effects of the DNA sequence (effect of the DNA or of its expression), expression of RNA, expression of proteins, amplification of hybridization probes for medical diagnosis, cell or gene therapy, vaccination, etc.

Please replace paragraph [0004] with the following heading and paragraph:

BACKGROUND

[0004] Recombinant vector construction, comprising insertion of a DNA fragment in a vector, includes an *in-vitro* recombinant vector reclosure step. In order for reclosure to take place, the ends of the finished vector must be in close proximity to each other. These ends move within a sphere with a radius equal to the length of the fragment and with one of the ends as its center. The greater the size of the finished vector, the greater the volume of this sphere. Consequently, the probability of conjunction of the two ends decreases with the length of the finished vector.

Please replace paragraph [0005] with the following paragraph:

[0005]Insertion of the fragment in the vector occurs if the probability of conjunction between the ends of the insert and those of the original vector is high. Thus, insertion is dependent on the concentration of ends.

Please replace paragraph [0007] with the following paragraph:

[0007] The very first generation vectors are vectors of bacterial origin derived from plasmids. Other vectors take into account more precisely the specificities of the eukaryote genes, in particular their size and have been developed to allow cloning of large pieces of DNA. This is the case for cosmids, hybrid compounds of phage l and plasmids reproducing in *E. coli,* and YACs whose host organism is yeast.

Please replace paragraph [0008] with the following paragraph:

[0008] The cosmids enable insertion up to 45 kb, the YACs up to 1000 kb. These vectors are intended for analysis of genomic DNA libraries or chromosomal analysis. Cosmids and YACs are responsive to the requirements of these analytical techniques and allow a search for a broad insertion range from DNA libraries. Their efficacy is linked to the range of different fragments that they can receive. Their use is burdensome if only insertion of a specific fragment and amplification of it are desired. Thus, in order to preserve their stability, the insertion vector must have a minimum size of 33 kb for the cosmids and 150 kb for the YACs. However, handling such lengths of DNA is fraught with numerous problems (breaks, cuts, etc.), and the sequence under consideration is frequently of smaller size, only reaching several kb when complementary DNA is preferred to genomic DNA. Moreover, replication in *E. coli* of vectors of large size such as cosmids as well as the stability of YACs in yeast are limited, which leads to sequence

modifications. In the case of the cosmids, the efficacy of the DNA packaging and of the infection by the phage l compensates for this disadvantage to a certain extent.

Please replace paragraph [0009] with the following paragraph:

[0009] Another insertion technique comprises the use of adjuvants of a chemical nature such as polyethylene glycol (Zimmerman S. B. and Pheiffer B. H., Proc. Natl. Acad. Sci. USA, 80, 5852 (1983)) or hexamine cobalt chloride (Maniatis et al., Molecular Cloning / A Laboratory Manual, second edition, 1989) for straight-end ligations. These ligations are not as easy to implement as cohesive-end ligations, and require higher concentrations of DNA and ligase. The purpose of these chemical adjuvants is to promote aggregation between insert and vector (intermolecular reaction) and to decrease the concentrations of DNA and ligase; these methods do not apply to the reclosure step (intramolecular ligation reaction) and to the construction of vectors of large size. The term "vector of large size" is understood to mean a vector of at least 10 kb into which is integrated an insert of multiple kilobases.

Please replace paragraph [0010] with the following paragraph:

[0010] The classical technique for construction of first generation vectors (E. coli derived plasmids) is effective for moderate sizes (several thousands of base pairs) but gradually loses its efficacy for larger vectors (more than 10kb). Moreover, at present, there is no technique which facilitates the circularization and preparation of vectors which is effective for vectors of moderate sizes -- several thousand of base pairs -- as well as for larger sizes -- more than 10 kb. Third, no known ligation adjuvants presently employed are proteins naturally designed to condense the DNA or have the effect of promoting the insertion of fragments of large size or reclosure of the recombinant.

Please delete paragraphs [0011] and [0012].

Prior to paragraph [0013], please insert:

BRIEF DESCRIPTION OF THE INVENTION

Please replace paragraph [0015] with the following paragraph:

[0015] In fact, one benefit of the invention is in the domain of cloning which generally includes a prior step of *in-vitro* cyclization of the recombinant. The closure of the recombinant fragment (linearized initial vector and insertion) can be meant as a DNA cyclization. Like DNA cyclization, the efficacy of vector closure depends on the size of the recombinant DNA fragment and is improved by DNA compaction.

Please insert the following paragraph [0016.1](formerly paragraph [0019]):

[0016.1] The phrase "by any suitable means" in step (b) should be understood to mean the transfer of the ligation products into a cellular medium suitable for DNA cloning, e.g., *E. coli* or yeast, in the presence of an antibiotic such as ampicillin or tetracycline, if the vector carries an antibiotic resistance gene, and a test for the presence of the insert, for example, in the case of the control gene lacZ, hydrolysis of the x-gal compound by the b-galactosidase produced.

Please replace paragraph [0017] with the following paragraph:

[0017] Thus, one application of the process of the invention is the cloning of an insert in a vector under the conditions defined above, advantageously enabling production of a recombinant vector of large size.

Please insert the following paragraphs (formerly paragraphs [0053]-[0057]):

[0018.1]Fig. 1 shows the complexation of the protein to the fragment fR4 under ligation conditions followed by retardation of the electrophoretic migration of the fragment in 0.4% agarose gel.

- 1a: Trial 1
- 1b: Trial 4
- 1c: Trial 1 with purified protein H1 (example 2)

[0018.2]Fig. 2 shows the digestion by EcoRI of the three recombinants R4-LZ with insert obtained in trial 11.

[0018.3] Fig. 3 shows the digestion by EcoRI of 6 of the 20 recombinants K-LZ (example 1b).

[0018.4] Fig. 4 shows the augmentation of the quantity of ligation products in the presence of histones, 0.4% agarose gel.

[0018.5] Fig. 5 shows the sensitivity test to the nucleases possibly present in the histone preparation.

Please delete paragraph [0019].

Please delete paragraph [0021].

Please replace paragraph [0022] with the following heading and paragraph:

DETAILED DESCRIPTION

[0022] According to the invention, ligation of the insert and of the original vector is implemented in the presence of DNA compacting products. The insertion vector contains a replication origin and, advantageously, a selection gene for growth and selection in the

cell type under consideration. According to one preferred implementation, the vector is a nonviral vector, albeit capable of containing viral elements.

Please replace paragraph [0024] with the following paragraph:

[0024] The DNA compacting products are proteins or any agents exhibiting the same properties, and more particularly the histone proteins or related proteins. Histones are the most abundant proteins in the nucleus; they are of small size (11 to 25 kDa) and of a very basic nature (pH > 10). There are five types of histones which are referred to as H1, H2A, H2B, H3 and H4, respectively. These five types are found with variants in all of the eukaryotes (with the exception of H1 which does not appear to exist in yeast, and which is replaced by histone H5 in certain organisms). H2A, H2B, H3 and H4 are found *in vivo* in octamer form. The DNA coils twice around the octameric core so as to form a nucleosomic structure. The histone H1 does not participate in this nucleosome core but serves to seal the DNA around the octamer. In the eukaryote cell, the compaction of the DNA by the histones has the effect, notably, of bringing close to each other two transcriptional regulation sites which are situated remotely from each other on the chromosome, and to enable formation of a chromatin loop by direct interaction between these sites. Thus, the expression of a gene can be controlled remotely by these genes (Amouyal, Biochimie (1991), 73, p. 1261-1268).

[0024.1] In addition to the histones, the DNA compaction agent can be selected from all the proteins known to compact DNA, especially the viral or phage envelope proteins, the bacterial chromoid proteins (HU, H-NS, etc.), the non-histone chromosomal proteins, the HMGs, etc., all mixtures of these compounds, or any derivative thereof.

Please delete paragraph [0026].

Please replace paragraph [0027] with the following paragraph:

[0027] The DNA compaction agent is preferably either a mixture of histones or a mixture of the aforementioned compaction agents.

Please replace paragraph [0028] with the following paragraph:

[0028] The inventor has demonstrated that a mixture of histones or an isolated histone lead to similar results. The histone H1, of different structure and not being a histone forming the octamer but rather a sealing histone, used by itself, however, does not appear to yield results as good as the other histones. This is explained by the fact that, because of its different structure, histone H1 does not bind to linear DNA but rather preferably to supercoiled DNA (Van Holde et al., Biophysical Journal, 1997, 72, p. 1388-1395).

Please delete paragraph [0029].

Please replace paragraph [0035] with the following paragraph:

[0035] This compaction agent concentration (C) can be expressed in milligrams (mg) of proteins per nanogram (ng) of total DNA contained in the ligation mixture per base pair (bp) of recombinant. The concentration is dependent on the length of DNA to be ligated and the inventor has defined a multiplicity of laws, each corresponding to the protein agents employed. Thus for the natural mixture of histones Sigma,

(C) = $1.5 \cdot 10^{-11}$ mg/ng DNA/bp recombinant

for the histone H2B Sigma,

(C) = $1.5 \cdot 10^{-12}$ mg/ng DNA/bp recombinant.

Please replace paragraph [0036] with the following paragraph:

[0036] By extrapolation, the inventor defined the following law (I) which can be applied to all of the compacting proteins employed:

$(C) = 10^{-x}$ mg/ng DNA/bp recombinant

in which x is between 8 and 15 inclusively.

The value x is a function of the nature of the compaction agent employed.

Please replace paragraph [0037] with the following paragraph:

[0037] In practice, the efficacy of the cloning is always improved if the concentration is within a range encompassed by the value defined above and extending from 20 to 1000% of that value, and preferably between 33 and 200% of this value.

Please replace paragraph [0038] with the following paragraph:

[0038] The process according to the present invention is thus characterized in that the concentration(C) of compaction agent is determined by the following law (II):

$(C) = (10^{-x}$ mg/ng DNA/bp recombinant) x Y

in which:

x is comprised between 8 and 15, preferably between 10 and 12, and

Y varies between 0.2 and 10, preferably between 0.33 and 2.

Please replace paragraph [0043] with the following paragraph:

[0043] Prior to ligation, the compaction agent is put into solution in the ligation buffer —possibly containing glycerol—(or diluted in the ligation medium, if the agent is provided in solution form). It is incorporated in the ligation medium in the proportions defined by the law specified above.

Please replace paragraph [0044] with the following paragraph:

[0044] In a preferred manner, the kit includes a stabilizing agent incorporated in the ligation medium at the same time as the compaction protein, which stabilizing agent is designed to prevent the denaturation, aggregation and/or adsorption of the compaction protein on the walls of the reaction tube at these strong dilutions. Thus, a kit according to the invention comprises:

- a ligation buffer,
- a ligase,
- a compaction agent,
- possibly a stabilizing agent for the protein.

Please replace paragraph [0045] with the following paragraph:

[0045] In a preferred manner, the stabilizing agent is glycerol or any compound presenting the same characteristics.

Please replace paragraph [0049] with the following paragraph:

[0049] The applications of the invention are, of course, to be found in the field of cloning, but also in the domain of gene therapy, most especially in the context of genetic vaccination. Thus, the invention pertains more specifically to a circularized recombinant nucleic acid of large size greater than several kilobases constituted by a vector and an insert. In a first form of implementation of the invention intended for gene therapy, said insert comprises one or more cDNA sequences coding for one or more proteins required for the correction of a genetic deficiency, placed under the control of sequences enabling their *in-vivo* expression.

Please replace paragraph [0050] with the following paragraph:

[0050] In a second form of implementation of the invention intended for genetic vaccination, said insert comprises one or more DNA sequences coding for one or more antigens, placed under the control of sequences enabling their *in-vivo* expression. More specifically, said insert comprises one or more, and preferably the totality, of the DNA sequences coding for antigens capable of inducing an immune reaction.

Please delete paragraph [0051].

Please delete paragraphs [0053]-[0057].

Please amend the heading after paragraph [0058] as follows:

Example 1a: Cloning a recombinant of 12,034 bp: Recombinant R4-LZ

Please replace paragraph [0062] with the following paragraph:

[0062] The 8,159-bp fragment (fR4) contains the replication origin colE1 and the ampicillin-resistance gene of *E. coli*. It is separated from the other fragments by electrophoretic migration on low-melting-point agarose gel (Seaplaque LMP agarose, FMC Bioproducts), excision and extraction of the corresponding band.

- Fragment of 3,875 base pairs: fLZ

Please replace paragraph [0064] with the following paragraph:

[0064] By digestion at the unique sites SpeI (position 29) and SalI (position 3904) followed by electrophoretic separation, the 3,875-bp fragment (fLZ)containing the gene lacZ under control of the bacterial promoter was separated from the 2,331-bp fragment.

Please replace paragraph [0068] with the following paragraph:

[0068] When necessary, the compaction protein is added to the mixture of fragments at the desired concentration. The T4 DNA ligase is added after incubation in a period of time between 0 to 20 minutes, more particularly between 3 and 5 minutes.

Please replace paragraph [0069] with the following paragraph:

[0069] The E.coli strain DH5a is a strain deficient in protein RecAl, which does not promote recombinations and rearrangements of the DNA within the cell (notably, the plasmids remain in monomer form while deletions are avoided, pages 4-13 of Maniatis).

Please replace paragraph [0071] with the following paragraph:

[0071] A first histone concentration value was determined by calculating the quantity of the natural mixture of histones assumed to be necessary for the formation of one nucleosome every 200 base pairs, and choosing deliberately to only take a part of the amount of protein thereby determined (one fourth in this example) so as to avoid saturation of the fragment in protein. Law III derived from this is the following:

(C) = $1.2 \cdot 10^{-11}$ mg of histone mixture/ng of DNA/bp of recombinant ...(III).

Please replace paragraph [0073] with the following paragraph:

[0073] The law, which was readjusted on the basis of these gels and which was subsequently employed, is very close to the calculated value:

(C) = $1.5 \cdot 10^{-11}$ mg of the histone mixture / ng of DNA / bp of recombinant ...(IV).

Please replace paragraph [0074] with the following paragraph:

[0074] Each ligation was performed in the presence of three concentrations of compaction proteins, the quantity derived from Law IV and quantities encompassing this quantity and corresponding to 50 and 200% of the calculated quantity, respectively.

Please replace paragraph [0075] with the following paragraph:

[0075] On these gels, we have:

- Track 1: Fragment fR4 without compaction proteins (except in the gel corresponding to trial 4),
- Track 2: Fragment fR4 + 0.025 μg of compaction protein,
- Track 3: Fragment fR4 + 0.05 μg of compaction protein,
- Track 4: Fragment fR4 + 0.1 μg of compaction protein,
- Track 5: Fragment fR4 + 0.2 μg of compaction protein (trial 4).

Please replace paragraph [0081] with the following paragraph:

[0081] The transformation of *E. coli* was implemented with the ligation mixture containing only one of these two fragments, in the presence of or absence of histones, to confirm the electrophoretic purity of the fragments and the absence of reclosure within the cell. The composition of the ligation mixture was analyzed prior to cellular transformation by standard gel electrophoresis. This analysis indicates that the addition of histones favors the obtention of ligation products prior to cellular transformation. For this purpose, the 20 μl of ligation mixture were deposited on a standard 0.4% agarose gel. The results are shown on Figure 4, in which:

- Track 1: ligation mixture (recombinant R4-LZ) without histones (fragments fR4-8159 bp and fLZ-3875 bp)

- Track 2: ligation mixture in the presence of 0.025 μg of histones
- Track 3: ligation mixture in the presence of 0.05 μg of histones
- Track 4: purified recombinant R4-LZ
- Track 5: 1-kb marker (Gibco BRL)
- Track 6: marker f_x174/HaeIII (Gibco BRL)

Please replace paragraph [0082] with the following paragraph:

[0082] The first 13 trials were performed on the basis of the quantity of compaction protein determined by gel retardation assay from the complexation of the fragment fR4 while they should have been performed on the basis of the complexation of the total recombinant (fR4 + fLZ) fragment. However, the size of the fragment fR4 represents only 2/3 of the total size of the recombinant. By employing these values for the ligations, only 2/3 of the value calculated according to the law was used (2/3 law). The more recent trials, which were also the more successful, were performed by taking into account the quantity of compaction protein (1/3 more, thus the exact value) that needed to be added to complex the entire vector and insert unit in the same manner as the fragment by itself.

Please replace paragraph [0085] with the following paragraph:

[0085] II.1. <u>2/3 Law (Trials 1-6, 9-13) versus Exact Law (Trials 14 and 15)</u>

- Trials 4 and 4': 4 quantities of compaction protein were tested: 50, 100, 200, 400%
- Trial 7: quantities of compaction protein 3 times higher than Trial 4 (150, 300, 600%)
- Trial 8: quantities of compaction protein 10 times higher than Trial 4 (500, 1000, 2000%)

(to compensate for a possible complexation deficit due to a lower concentration of DNA)

- Trials 14 and 15: exact law

Please replace paragraph [0086] with the following paragraph:

[0086] II.2. Effect of storage and freezing of the compaction protein:

- Trial 2: dilution of the compaction protein from a stock-solution of 100 mg/ml in the dilution buffer containing 50% glycerol, stored at -20°C to test the effect of storage. For all of the other trials, the protein solution was freshly prepared from the lyophilized powder.

Please replace paragraph [0090] with the following paragraph:

[0090] II.6. Presence of glycerol in the dilution buffer

- Trials 9, 10, 11', 12', 12''', 14, 15

Glycerol is usually added to the storage buffer, as above, to prevent denaturation and aggregation of proteins during successive freezing-thawing cycles. Thus, 0.1% of glycerol was added to the compaction protein dilution buffer (ligation buffer) so as to prevent aggregation or denaturation of the compaction protein when it was put into solution, as well as to avoid the loss of compaction protein on the tube walls in the final dilutions. In trial 11', glycerol was added on a delayed basis (after the first ligation series of trial 11, i.e., after approximately ten minutes).

Please insert paragraph [0090.1] (formerly paragraph [0096]):

[0090.1] The presence of glycerol when the protein is put into solution appears to be beneficial to the cloning efficacy in the case of the natural histone mixture. It does not

appear to be especially beneficial with the isolated histones (H1 or H2B, example 2). That is why the kit can contain (irrespective of the protein employed) a dilution buffer containing glycerol (or any other agent with the same properties) as well as a dilution buffer without glycerol (which is also the ligation buffer).

Please replace paragraph [0092] with the following paragraph:

[0092] In each trial, recombinants not possessing the anticipated sequence by cloning in *E. coli* were selected. These defects are generated at the time of *in vitro* ligation or by incorrect replication of the DNA by the cell.

Please replace paragraph [0094] with the following paragraph:

[0094] In order to determine whether the presence of these incorrect recombinants is due specifically to the use of the compaction protein preparation, especially to the possible presence of nucleases, and to the partial degradation of the DNA which could result from it, a DNA sensitivity test to the nucleases in the histone preparation employed was performed. For this test, the fragment fR4 or the plasmid pR4 was incubated with the quantities of protein indicated on tracks 3 to 10. The results are illustrated in figure 5, in which:

- Track 3: fragment fR4 (500 ng)
- Track 4: fragment fR4 + 0.025 μg of histones
- Track 5: fragment fR4 + 0.05 μg of histones
- Track 6: fragment fR4 + 0.1 μg of histones
- Track 7: plasmid pR4 (500 ng)
- Track 8: plasmid pR4 + 5 μg of histones
- Track 9: plasmid pR4 + 0.025 μg of histones

– Track 10: plasmid pR4 + 0.05 μg of histones

– Tracks 11, 12, 13: like tracks 8, 9, 10. Incubation at 20°C for 1.5 hours rather than 20 hours.

– Tracks 1 and 14: 1-Kb marker (Gibco BRL)

– Track 2 and 15: marker f_x174/HaeIII (Gibco BRL)

No nuclease digestion was detected under these conditions.

Please delete paragraph [0096].

Please replace paragraph [0098] with the following paragraph:

[0098] Example 1b: Cloning of a 6,785-bp recombinant: Recombinant K-LZ

I – Material and Methods

I.1 Fragments

- Fragment of 2910 base pairs: fK.

Please replace paragraph [0102] with the following paragraph:

[0102] The quantities of compaction protein employed corresponded to 33, 67 and 134% of the quantity indicated by the law specified above (2/3 law).

Please replace paragraph [0104] with the following paragraph:

[0104]The usual technique is still valid for this 6,785 bp construct, in the absence of histones, while it was not anymore for the larger recombinant R4-LZ, under the same condition. However, the addition of histones also increases the cloning efficacy.

Please replace paragraph [0111] with the following paragraph:

[0111] Example 2b: ligation in the presence of histone H2B

In this case, the protein (M = 13,774) represents one tenth of the mixture (M = 130,268), and the law becomes:

(C) = $1.5 \cdot 10^{-12}$ mg H2B/ng DNA/bp of recombinant.

In the Claims (Clean Copy as Amended)

Please cancel Claims 1-10 without prejudice and without disclaimer of the subject matter contained therein.

Kindly add the following new claims:

11. (New) A process for preparing circularized recombinant nucleic acids from a vector and an insert comprising the steps of:

producing circularized recombinant nucleic acid by ligating a DNA insert and a DNA vector in the presence of a DNA compaction agent;

and

selecting said circularized recombinant nucleic acid.

12. (New) The process according to Claim 11, wherein the size of said circularized recombinant nucleic acid is greater than 10 kb.

13. (New) The process according to Claim 11, wherein said selection comprises the steps of:

transferring said circularized recombinant nucleic acid into a cellular medium,

cloning said circularized recombinant nucleic acid, and

testing for presence of said insert in said circularized recombinant nucleic acid.

14. (New) The process according to Claim 11, wherein said DNA compaction agent is one selected from the group consisting of a protein, a mixture of proteins, and protein derivatives.

15. (New) The process according to Claim 11, wherein said DNA compaction agent is one selected from the group consisting of histone proteins, histone protein derivatives, viral envelope proteins, phage envelope proteins, bacterial chromoid proteins, non-histone chromosomal proteins, HMGs, derivatives of said proteins, and mixtures of said proteins and protein derivatives.

16. (New) The process according to Claim 11, wherein said ligation comprises the step of adding a ligase to a ligation medium comprising DNA in solution in ligation buffer.

17. (New) The process according to Claim 16, wherein said DNA compaction agent is added to said ligation medium prior to the addition of said ligase.

18. (New) The process according to Claim 16, wherein said DNA compaction agent is added to said ligation medium simultaneously with the addition of said ligase.

19. (New) The process according to Claim 11, wherein said DNA compaction agent is present at a concentration (C) allowing flexibility of said DNA.

20. (New) The process according to Claim 19, wherein said DNA compaction agent concentration (C) is defined by the following equation:

$$(C) = 10^{-x} \text{ mg DNA compaction agent/ng DNA/bp recombinant,}$$

wherein X = 8-15.

21. (New) The process according to Claim 19, wherein said DNA compaction agent concentration (C) is defined by the following equation:

$$(C) = (10^{-x} \text{ mg DNA compaction agent/ng DNA/bp recombinant}) \times Y$$

wherein X = 8-15 and

Y = 0.2 - 10.

22. (New) The process according to Claim 16, wherein said ligation medium further comprises a stabilizing agent, wherein said stabilizing agent is capable of preventing denaturation, aggregation, and absorption of said DNA compaction agent.

23. (New) The process according to Claim 15, wherein said histone proteins are selected from the group consisting of histone H1, H2A, H2B, H3, and H4.

24. (New) A kit for preparing circularized recombinant nucleic acids from a vector and an insert comprising a ligation buffer, a ligase, and a DNA compaction agent.

25. (New) The kit according to Claim 24, wherein said DNA compaction agent is one selected from the group consisting of histone proteins, viral envelope proteins, phage envelope proteins, bacterial chromoid proteins, non-histone chromosomal proteins, HMGs, derivatives of said proteins, and mixtures of said proteins and protein derivatives.

26. (New) The kit according to Claim 25, wherein said histone proteins are selected from the group consisting of histone H1, H2A, H2B, H3, and H4.